学　位　論　文

# Cholesteryl-palmitate crystals in bronchoalveolar lavage fluid smears as a possible prognostic biomarker for choronic interstitial pneumonia : A preliminary study

（　慢性間質性肺炎における BAL 液スメアのパルミチン酸結晶の予後予測因子としての可能性　）

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# Cholesteryl palmitate crystals in bronchoalveolar lavage fluid smears as a possible prognostic biomarker for chronic interstitial pneumonia: A preliminary study

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ABSTRACT

*Background:* We observed cholesterol-like crystals (Crystal X) in the bronchoalveolar lavage fluid (BALF) smears of patients with diffuse pulmonary disease. We analyzed the clinical data of patients with and without crystals, and elucidated the structure of Crystal X and its concentration in the BALF.

*Methods:* Two hundred eighty-nine patients with diffuse pulmonary disease who underwent bronchoalveolar lavage (BAL) were analyzed. The relationships between the presence and number of Crystal X in BALF smears and clinical parameters were investigated. Furthermore, structure determination and quantitative analyses of the crystals were performed.

*Results:* Seventy-five (26.0%) patients had Crystal X in their BALF. The crystals were frequently observed in patients with chronic interstitial pneumonia (CIP, 60/160=35.3%). Patients with Crystal X exhibited significantly higher serum Kerbs von Lungren 6 antigen and surfactant protein-D levels ($P<0.01$) and lower percentage vital capacity ($P<0.05$) than patients without Crystal X. The number of crystals was significantly correlated with these parameters. The presence of crystals was also associated with a lower survival rate at 1 year after the BAL. The interfacial angles of the crystals were $126\pm2^{\circ}$ and $144\pm2^{\circ}$, different from those of cholesterol monohydrate crystals. Infrared absorption spectrometry showed Crystal X was cholesteryl palmitate. Its concentration was significantly higher in BALF with crystals than in BALF without crystals ($P<0.01$).

*Conclusions:* Crystal X in the BALF of patients with diffuse pulmonary disease was identified as cholesteryl palmitate, which may be a useful prognostic biomarker for CIP.



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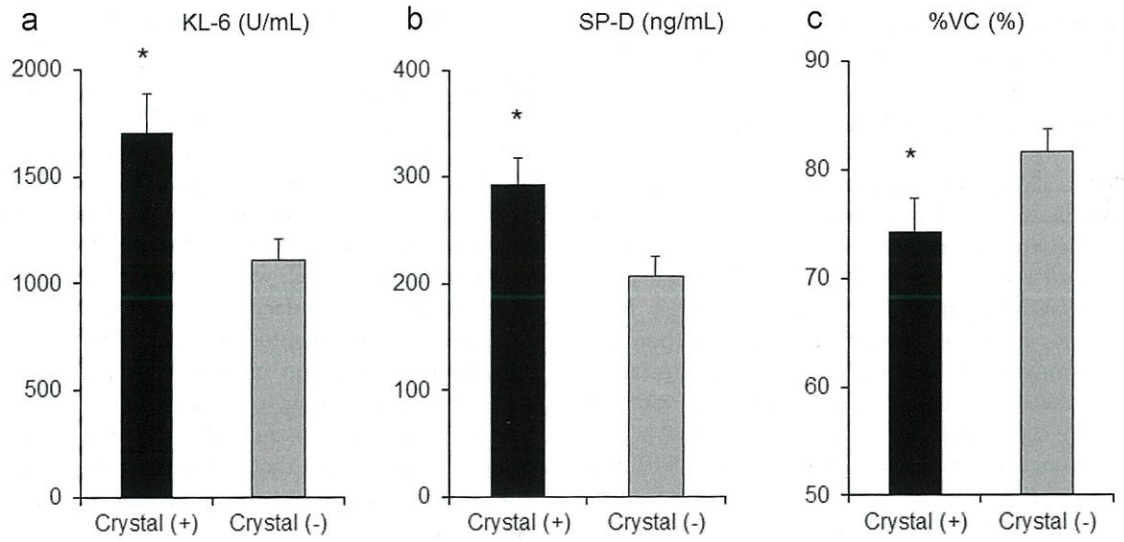


**Fig. 2 – The comparison of the clinical parameters of patients with chronic interstitial pneumonia with and without cholesterol-like crystals (Crystal X) in the bronchoalveolar lavage fluid (BALF) smears. The comparison between patients with chronic interstitial pneumonia with and without Crystal X in the BALF smears reveals significantly higher serum levels of KL-6 and SP-D ($P<0.05$) and a significantly lower %VC value ($P<0.05$) in the Crystal X (+) group than in the Crystal X (−) group. The mean and standard error (SE) values are plotted. KL-6, Kerbs von Lungren 6 antigen; SP-D, surfactant protein-D; %VC, percentage vital capacity.**

A piece of crystal in the BALF smear was obtained and its Fourier transform infrared spectrum was measured with transmitted light (Herschel FT/IR-660; JASCO Corp., Tokyo, Japan) using the JASCO Irtron IRT-30 microscope unit (JASCO Corp.). The cumulative number was 100 and the resolution was $4\,cm^{-1}$. An MCT detector was used as the infrared detection element device. The IR Standard Database (Bio-Rad Sadtler, Hercules, CA) was used to identify Crystal X.

For the quantitative analysis using HPLC, 3 mL of chloroform and cholesteryl benzoate (i.e., the internal standard) were added to the BAL supernatant (0.3 mL). The chloroform layer was separated by shaking the mixture well for 5 min. The extracted solution was dried with anhydrous sodium sulfate, and the solvent was removed in vacuo. The residue was dissolved in hexane and analyzed by HPLC (Model LC-1500; JASCO Corp.). The analysis conditions were as follows: 15-cm column (Chiralcel OD-H; Daicel Corp., Tokyo, Japan); detector, ultraviolet (200 nm); eluent, methanol/isopropanol/water mixture (gradient, 96/0/4 for 0–4 min and 70/30/0 after 4 min); and flow rate, 1 mL/min at 25 °C.

### 2.6. *Statistical analysis*

The clinical parameters of the patients with and without Crystal X in the BALF smears were compared using the Student t test. The crystal (+) and crystal (−) subgroups were compared using the chi-square test. Spearman's rank correlation analysis was used to determine the correlation between the clinical parameters and the number of Crystal X. The Kruskal–Wallis test was used to compare the number of Crystal X and the frequency of crystal-positive results between patients with IPF, those with other types of IIP, and those with IP-CTD. The Mann–Whitney test was used to compare the concentration of dissolved Crystal X in the BAL supernatant. All data were expressed as the mean ± standard error (SE), unless otherwise specified. A value of $P \leq 0.05$ was accepted as significant.

## 3. Results

### 3.1. *The presence of Crystal X in the BALF smears of patients with various forms of diffuse pulmonary disease*

During the study period, 289 patients with diffuse pulmonary disease underwent BAL (Table 1). Crystal X in the BALF smears was observed in 45 of 130 patients with IIP, 15 of 40 patients with IP-CTD, four of 38 patients with sarcoidosis, four of seven patients with hypersensitivity pneumonia, three of 11 patients with organizing pneumonia, two of the three patients with alveolar proteinosis, one of the 11 patients with pneumocystis pneumonia, and one of three patients with radiation pneumonitis. There was no Crystal X in the BALF smear among patients with drug-induced pneumonia ($n=10$), eosinophilic pneumonia ($n=10$), pneumonia ($n=7$), or other diseases ($n=19$). The comparison between the CIP and non-CIP groups showed that the frequency of the presence of Crystal X was significantly higher in the CIP group (60/170 [35.3%] patients) than in the non-CIP group (15/119 [12.6%] patients); ($P<0.001$). Only three patients had alveolar proteinosis; however, they had a high number of crystals in the BALF smear.

### 3.2. *The association between the presence of Crystal X and the clinical parameters in patients with CIP*

Among 170 patients with CIP, 60 patients (45 patients with IIP and 15 patients with IP-CTD) had Crystal X in the BALF

smears, while the remaining 110 patients (85 patients with IIP and 25 patients with IP-CTD) did not. The clinical data were available from 148 patients. These data were compared between the crystal (+) subgroup ($n=54$) and the crystal (−) subgroup ($n=94$). The crystal (+) subgroup had significantly higher serum levels of Kerbs von Lungren 6 antigen (KL-6) (1704±187 U/mL vs. 1112±97 U/mL) and surfactant protein-D (SP-D) (292.8±24.8 ng/mL vs. 206.5±17.9 ng/mL) ($P<0.01$ for both) and a significantly lower percentage vital capacity (% VC) value (74.4%±3.0% vs. 81.7%±2.1%) ($P<0.05$, Fig. 2) than the crystal (−) subgroup.

### 3.3. *The association between the number of Crystal X in the BALF smears and the clinical parameters of patients with CIP*

The correlation between the number of Crystal X in the BALF smears and the clinical parameters was analyzed. A significantly positive but weak correlation existed between the number of Crystal X and the serum levels of lactate dehydrogenase, KL-6, and SP-D. With regard to the parameters of the BALF, the total cell count and the number of neutrophils had a positive correlation with the number of Crystal X. A negative correlation existed between the number of Crystal X and the %VC value (Table 2). Patients with IPF ($n=49$), other types of IIP ($n=81$), and IP-CTD ($n=40$) showed no difference in the number of Crystal X (3.63±0.7, 8.81±3.2, and 17.6±6.8, respectively) and the frequency of crystal (+) (19/49 patients, 26/81 patients, and 15/40 patients, respectively). The outcomes were analyzed at 1 year after the BAL among the 121 patients for whom follow-up data were available. The survival rate was 76.1% (35/46 patients) in the crystal (+) subgroup and 89.3% (67/75 patients) in the crystal (−) subgroup, which indicated a significantly poorer outcome in the crystal (+) subgroup ($P=0.05$).

**Table 2 – Relationship between the number of Crystal X and clinical parameters.**

| | Correlation coefficients | P value |
|---|---|---|
| Age (years) | −0.14 | 0.07 |
| WBC (per microliter) | −0.02 | 0.75 |
| LDH (U/mL) | 0.17 | <0.05 |
| Cholesterol (mg/dL) | 0.06 | 0.47 |
| CRP (mg/dL) | 0.03 | 0.70 |
| ESR (mm/h) | −0.01 | 0.85 |
| KL-6 (U/mL) | 0.34 | <0.001 |
| SP-A (ng/mL) | 0.00 | 0.99 |
| SP-D (ng/mL) | 0.32 | <0.001 |
| Total cell count in BALF ($\times 10^4$/mL) | 0.17 | <0.05 |
| AM in BALF ($\times 10^4$/mL) | 0.06 | 0.50 |
| Lym in BALF ($\times 10^4$/mL) | 0.11 | 0.15 |
| Neu in BALF ($\times 10^4$/mL) | 0.16 | 0.05 |
| Eos in BALF ($\times 10^4$/mL) | −0.01 | 0.95 |
| %VC (%) | −0.15 | 0.06 |
| $FEV_1$% (%) | 0.14 | 0.07 |
| $\%DL_{co}$ (%) | −0.14 | 0.11 |

AM, macrophages; BALF, bronchoalveolar lavage fluid; CRP, C-reactive protein; $DL_{co}$, diffusing capacity for carbon monoxide; Eos, eosinophils; ESR, erythrocyte sedimentation rate; $FEV_1$, forced expiratory volume in 1 s; KL-6, Krebs von den Lungen 6; LDH, lactate dehydrogenase; Lym, lymphocytes; Neu, neutrophils; SP-A, surfactant protein-A; SP-D, surfactant protein-D; %VC, percentage vital capacity; WBC, white blood cell.

### 3.4. *Analysis of Crystal X*

Crystal X in the BALF smears (Fig. 1a–c) were polygonal crystals, and differed in appearance from cholesterol monohydrate crystals. The cholesterol monohydrate crystals in the urine of patients with nephrotic syndrome usually assume the form of parallelograms and one angle is characteristically missing in some of the crystals (Fig. 1d). However, Crystal X was polygonal with five or more angles, and clearly differed in appearance from cholesterol crystals. The interfacial angles were 124±2° and 144±2°, which were equal to the angles in a cholesteryl palmitate crystal and clearly different from the angles of a cholesterol monohydrate crystal (79.15° and 100.85°) [7]. The infrared absorption spectrum of Crystal X showed an absorption band at 1743 $cm^{-1}$, which corresponded to an ester group, and was identical with the absorption band of cholesteryl palmitate (Fig. 3). On the basis of the aforementioned results, Crystal X was identified as cholesteryl palmitate crystals. The results of quantitative analysis by HPLC showed that concentrations of cholesteryl palmitate in the BALF were 0.580±0.257 μg/mL in the crystal (+) subgroup ($n=6$) and 0.032±0.019 μg/mL in the crystal (−) subgroup ($n=4$) (Fig. 4), which indicated a significantly higher content in the crystal (+) subgroup ($P<0.01$).

## 4. Discussion

In the present study, we focused on Crystal X in the BALF smears of the patients with diffuse pulmonary disease and obtained the following results. Crystal X was frequently present in patients with CIP. Among patients with CIP, those with Crystal X had significantly higher serum lactate dehydrogenase, KL-6, and SP-D levels, and the number of Crystal X in the BALF smears were correlated with the serum levels of these parameters. At 1 year after the BAL, the survival rate was significantly lower in the patients with Crystal X. Infrared absorption microspectrometry and interfacial angles measurement of Crystal X revealed that the crystals were actually cholesteryl palmitate crystals. The concentration of cholesteryl palmitate in the BALF was significantly higher in patients with crystals, compared to patients without them.

To the best of our knowledge, this is the first study to report the presence of cholesteryl palmitate crystals in BALF smears. In 1968, Glancy et al. [8] reported 12 autopsy cases of pulmonary hypertension (including two cases of diffuse interstitial lesions) with granulomas that contained cholesterol-like crystals. They performed an X-ray crystallographic analysis of a granuloma from one patient, and revealed that the crystalline material was not free cholesterol but was cholesteryl palmitate, cholesteryl stearate, or a mixture of the two. Villalba et al. [9] recently measured the serum lipid profiles in patients with pulmonary diseases by using liquid chromatography–tandem mass spectrometry. They reported that patients with IPF or familial interstitial

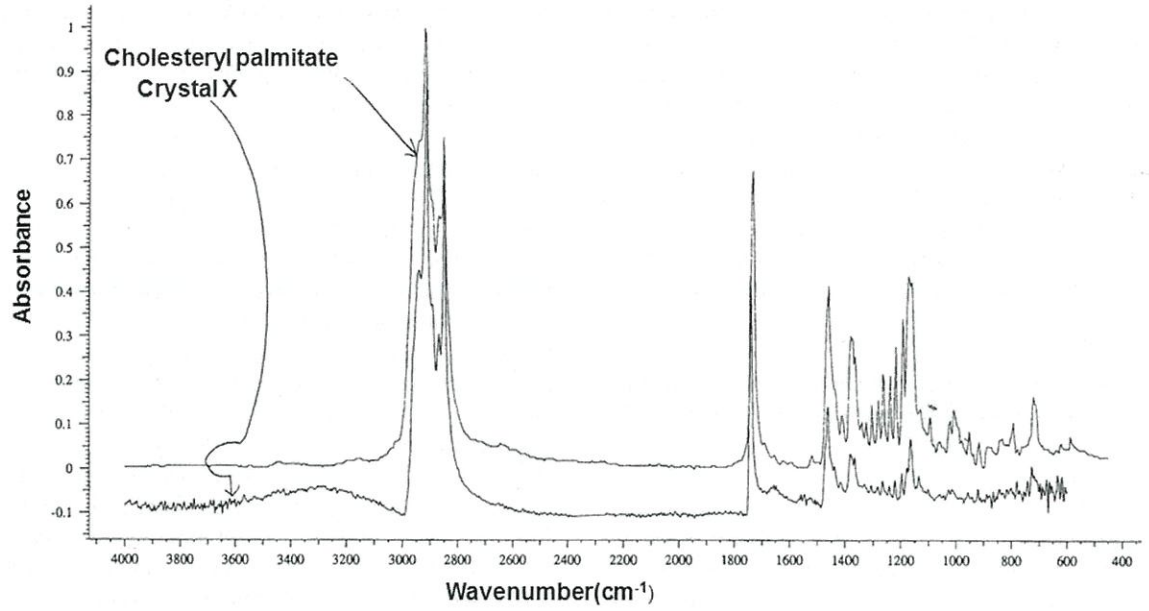


**Fig. 3 – The infrared spectra of cholesterol-like crystals (Crystal X) and cholesteryl palminate. The spectrum of Crystal X and cholesteryl palmitate is identical.**

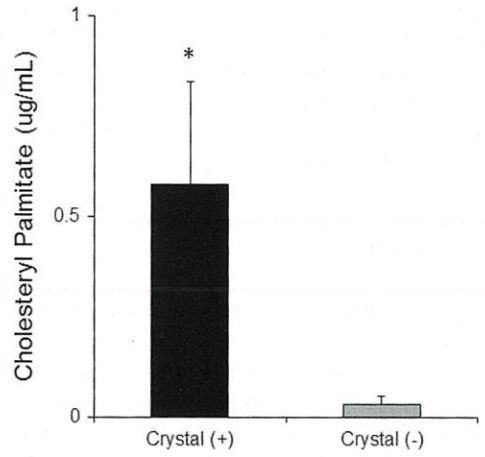


**Fig. 4 – Quantitative analysis of cholesteryl palmitate in the bronchoalveolar lavage fluid (BALF) by high-performance liquid chromatography. The concentration of cholesteryl palmitate in the BALF is significantly higher in the cholesterol-like crystal-positive [crystal X (+)] group ($n=6$) than in the crystal X (−) group ($n=4$) ($P<0.05$). The mean and standard error (SE) values are plotted.**

pneumonia (IP) had higher serum levels of cholesteryl palmitate than healthy individuals. Fireman et al. [10] reported two patients with IPF who had crystal structures in their BALF smears and concluded that they were cholesterol crystals. However, the interfacial angles of the crystals represented in the figure in their article seemed to be different from the angles of cholesterol monohydrate crystals. Thus, we believe that the crystals may have been cholesteryl palmitate crystals, as in the present study.

Pulmonary surfactant is a complex that consists of lipids and proteins at a ratio of 9:1. Dipalmitoyl phosphatidylcholine, a phospholipid with two palmitic acids molecules, accounts for approximately 50% of the phospholipids in pulmonary surfactant [11] and it has a central role in surfactant activity [12]. Phosphatidylcholine has various acyl chains and the composition of these chains are altered in patients with diffuse pulmonary diseases and acute respiratory distress syndrome [13,14]. Fatty acids that constitute the pulmonary surfactant lipids are synthesized from carbohydrates and proteins via acyl-CoA. The synthesis ends at palmitic acid (C16). Stearic acid and oleic acid are thereafter endogenously synthesized through C16 fatty acid by elongation of very long chain fatty acid member 6 (Elovl6). Sunaga et al. [15] recently reported that Elovl6 expression was significantly decreased in the lungs of patients with IPF. They also reported an increase in the palmitic acid level in Elovl6-deficient mice and its association with increased oxidative stress and pulmonary fibrosis. On the other hand, cholesterol esters such as cholesteryl palmitate have an important role in lipid transport in the body.

In recent years, the role of ATP-binding cassette transporter A (ABCA) in lipid transport has been clarified. The molecule ABCA1 is involved in the elimination of cholesterol from the lung and its transport to the liver. In ABCA1-knockout mice, an increase in the amount of cholesterol in the lung and a considerable increase in the amount of cholesterol esters in the alveolar macrophages are observed with pathological findings similar to alveolar proteinosis [16]. Furthermore, ABCA3 has been shown to be involved in the transport of surfactant phospholipids [17]. Several studies have also reported an association between mutations of the *ABCA3* gene and neonatal IP and adult IP [17–20].

The presence of cholesteryl palmitate crystals in the BALF smears in our study may be associated with an increase in the content of cholesteryl palmitate in the alveoli and may reflect abnormal lipid metabolism in patients with IP, as described previously. The results of the present study, which include high serum levels of IP markers (e.g., KL-6 and SP-D), low %VC, and poor outcomes in patients with BALF

cholesteryl palmitate crystals, lend support to the aforementioned notion.

## 5. Conclusions

This preliminary study revealed that cholesteryl palmitate crystals are present in BALF smears and may be a possible prognostic biomarker in patients with diffuse pulmonary disease. In addition, the presence of cholesteryl palmitate crystals seemed to be associated with the pathophysiology of CIP. In the future, it would be worthwhile to conduct a prospective clinical study with quantitative analysis of cholesteryl palmitate in BALF, and to promote basic research on the association between fatty acid esters such as cholesteryl palmitate and pulmonary inflammation and fibrosis.

## Conflict of interest

The authors have no conflicts of interest.

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